FEBS Letters 582 (2008) 881–885

PKC and MEK pathways inhibit caspase-9/-3-mediated cytotoxicity in differentiated cells

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Received 8 January 2008; revised 31 January 2008; accepted 6 February 2008

Available online 20 February 2008

Edited by Lukas Huber

Abstract **Many studies have indicated that differentiated cells inhibit drug-induced cytotoxicity but undifferentiated cells do not, though the mechanisms are unclear. Currently, HL-60 cells are induced to differentiate into macrophage-like cells with Phorbol-12-myristate-13-acetate (TPA) treatment (TPA-differentiated cells). Our study shows that caspase-9/-3-mediated cytotoxicity can be induced in undifferentiated HL-60 cells but not in TPA-differentiated HL-60 cells. However, caspase-9/-3-mediated cytotoxicity can be induced in TPA-differentiated cells if they are pretreated with a protein kinase C (PKC) or a mitogen activated protein kinase (MEK) inhibitor. Taken together, this study demonstrates that TPA-differentiated HL-60 cells inhibit caspases-9/-3-mediated cytotoxicity through the PKC and MEK signaling pathways.**

Keywords: Caspases; PKC; MEK; Differentiation

1. Introduction

RC-RNase belongs to RNase A superfamilies, having RNase activities and is derived from oocytes of bull frog [1,2]. RC-RNase, a 13 kD protein, has a lectin domain which may be associated to tumoricidal effect [2,3]. Many studies demonstrated that RC-RNase has anti-tumor effects [1–3] and RC-RNase-induced cytotoxicity was shown to correlate with cell differentiation [4,5]. A study conducted by Hu et al. showed that RC-RNase exerted a strong tumoricidal activity on poorly differentiated hepatoma cells compared to a lower activity on well-differentiated hepatoma cells [4]. A similar study also showed that RC-RNase-induced cytotoxicity and caspase-9/-3 activities on HL-60 cells but not on retinoic acid (RA)-differentiated-HL-60 cells [5]. This study aims to further test whether RC-RNase-induced caspase-9/-3-mediated cytotoxicity is inhibited on TPA-differentiated cells.

The HL-60 cell line is a good system for studying differentiation. HL-60 cells can be induced to differentiate into granulocytes or macrophage-like cells following pretreatment with different factors, i.e., TPA pretreatment [6,7]. TPA is a differentiating agent that induced HL-60 cells to differentiate into macrophage-like cells (TPA-differentiated cells) [8,9]. To determine whether TPA-differentiated HL-60 cells inhibit RC-RNase-induced cytotoxicity, HL-60 cells and TPA-differentiated HL-60 cells are treated with RC-RNase. Data from our study showed that RC-RNase induces caspase-9/-3 activation and cytotoxicity on HL-60 cells, but not on TPA-differentiated HL-60 cells. Many studies also showed that TPA can induce HL-60 cells to differentiate into macrophage-like cells through protein kinase C (PKC) and mitogen activated protein kinase (MEK) pathways [10–12]. To examine whether PKC and MEK pathways inhibit RC-RNase-induced cytotoxicity, PKC inhibitor and MEK inhibitor are used in this study. Our data showed that RC-RNase cannot directly induce cytotoxicity on TPA-differentiated HL-60 cells. However, RC-RNase can induce cytotoxicity on TPA-differentiated HL-60 cells when it is pretreated with bisindolylmaleimide I or PD98059. This study then indicates that RC-RNase-induced cytotoxicity is inhibited on HL-60 cells through PKC and MEK pathways.

Previous studies demonstrated that the MEK pathway could inhibit caspase-8-mediated cell death [13,14]. However, whether the MEK pathway can inhibit caspase-9 activation is still unclear. In this study, RC-RNase induces caspase-9/-3 activation on HL-60 cells while RC-RNase cannot induce caspase-9/-3 activation on TPA-differentiated HL-60 cells. Furthermore, RC-RNase can only induce caspase-9/-3 activation on TPA-differentiated HL-60 cells after pretreatment with bisindolylmaleimide I or PD98059. This data suggests that the PKC or the MEK pathway can inhibit caspase-9/-3 activation. Overall, this study demonstrates that

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0014-5793/$34.00
doi:10.1016/j.febslet.2008.02.018

TPA-differentiated HL-60 cells inhibit RC-RNase-induced caspases-9/-3 activation and cytotoxicity through the PKC and the MEK pathways.

2. Materials and methods

2.1. Chemicals and cell culture

TPA, RA and nitroblue tetrazolium (NBT) were purchased from Sigma. DMSO was provided by Merck. Bisindolylmaleimide I and PD98059 were obtained from Calbiochem. Ac-DEVD-pNA (caspase-3 substrate) and Ac-LEHD-pNA (caspase-9 substrate) were procured from Anaspec. HL-60 cells were cultured in RPMI1640 medium, supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids (Gibco).

2.2. Purification

RC-RNase could be purified [1,2]. Briefly, RC-RNase was extracted from yolk granules. The extract was centrifuged and the soluble fraction was dissolved in PC buffer (20 mM HEPES, pH 7.9, 0.1 mM EDTA). The supernatant was loaded onto a phosphocellulose column, and the fractions containing RC-RNase were identified by CpG cleavage assay [1] and Ribonuclease activity assay [15]. These active fractions were collected and loaded onto a carboxymethyl cellulose column. The fractions containing RC-RNase activity were analyzed by SDS–PAGE. The purity of RC-RNase was determined by the Bradford method [16].

2.3. Differentiation and adhesion assay

A solution containing 100 μl of 5×10^4/ml cells was seeded on a 96-well dish and treated with 20 nM TPA for 1 day (TPA was dissolved in DMSO, final concentration of DMSO in media was less than 0.02%). Control cells were treated with a similar concentration of DMSO. To examine the role of PKC and MEK pathways, 2 μM PKC inhibitor, bisindolylmaleimide I and 25 μM MEK inhibitor, PD98059, were added to the media, respectively, 30 min prior to the addition of TPA. The numbers of TPA-differentiated cells were determined using an adhesion assay [11]. Briefly, cells were fixed with 10% formalin for 10 min and incubated at 37 °C for 30 min after addition of 0.5% crystal violet (in 20% methanol) and subsequently washed. After washing, the stain was eluted with 0.1 M sodium citrate (pH 4.2, in 50% ethanol) and was measured at 550 nm in an ELISA reader (Molecular Devices).

2.4. RA-differentiated cells and NBT assay

HL-60 cells were induced to differentiate into granulocytes (RA-differentiated cells) by treating with 1 μM RA for 5 days. The number of RA-differentiated cells were determined by NBT assay [5]. Briefly, HL-60 cells were incubated at 37 °C for 40 min after addition of 0.2% NBT containing 200 ng/ml TPA. Intracellular blue formazan deposits could be detected in differentiated cells. A minimum of 200 cells were counted.

2.5. Cell proliferation assay

Approximately, 10^5/ml cells (containing HL-60 cells and TPA-differentiated cells, HL-60 cells pretreated with PD98059, HL-60 cells pretreated with bisindolylmaleimide I, TPA-differentiated cells pretreated with PD98059 and TPA-differentiated cells pretreated with bisindolylmaleimide I) were treated with 20 μg/ml RC-RNase. Cell proliferation was determined daily using a hemocytometer with tryptophan blue stain.

2.6. Caspase activity assay

Cell lysates were obtained by treating cells with a lysis buffer (50 mM Tris–HCl; 120 mM NaCl; 1 mM EDTA; 1% NP-40, pH 7.5) supplemented with protease inhibitors (Calbiochem). Caspase assay were performed by pipetting 40 μl cell lysates to a 96-well dish, containing 158 μl reaction buffer (20% glycerol; 0.5 mM EDTA; and 5 mM DTT; 100 mM Hepes, pH 7.5), and 2 μl fluorogenic Ac-DEVD-pNA or Ac-LEHD-pNA. Samples were incubated for 8 h at 37 °C and determined at 405 nm in an ELISA reader (Molecular Devices). The increasing fold of caspase activity was indicated as $A405_{(\text{RC-RNase treatment})}/A405_{(\text{control: without RC-RNase treatment})}$. This method has been described previously [3,5,17].

3. Results

3.1. Successful induction of differentiation on HL-60 cells with TPA treatment

After TPA treatment for 1 day, undifferentiated HL-60 cells were induced to differentiate into macrophage-like cells (TPA-differentiated cells). Undifferentiated cells and TPA-differentiated cells could be easily determined by an adhesion assay. Undifferentiated (suspension) cells were washed out prior to adhesion assay. Thus, no cells were stained (Fig. 1A). An O.D. value close to 0 was observed in the adhesion assay (Fig. 1C). TPA-differentiated (attached) cells are stained with crystal violet (Fig. 1B). Here, an O.D. value of nearly 2 was observed (Fig. 1C). To test the role of the PKC and MEK pathway, a PKC inhibitor (bisindolylmaleimide I) and a MEK inhibitor (PD98059) were used to pretreat the cells. Again, an O.D. value close to 0 was observed in the adhesion assay (Fig. 1C). Hence, the data indicate that TPA induces HL-60 cells differentiation through the PKC and MEK pathways.

3.2. TPA-differentiated cells inhibit RC-RNase-induced cytotoxicity

As shown in Fig. 2A, RC-RNase induces cytotoxicity which leads to a decrease in the number of undifferentiated cells. Though growth arrest was found in TPA-differentiated cells, there was no significant difference in cell number between TPA-differentiated cells without RC-RNase treatment and those with RC-RNase treatment (Fig. 3A). Thus, TPA-differentiated cells inhibit RC-RNase-induced cytotoxicity while undifferentiated cells do not. Additionally, bisindolylmaleimide I and PD98059 were used to test whether MEK and PKC pathways could inhibit RC-RNase-induced cytotoxicity. Our data show that RC-RNase does not decrease the cell num-

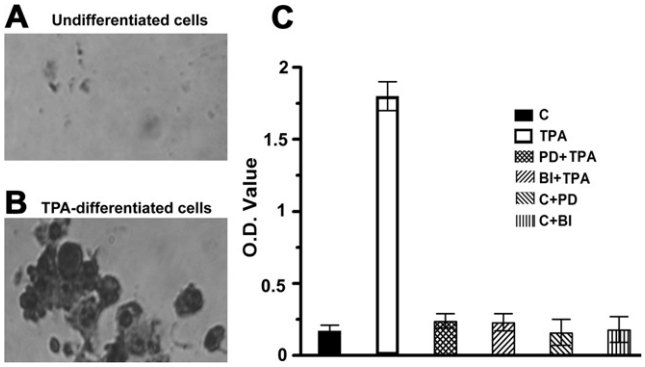

Fig. 1. TPA induces HL-60 cells differentiation through PKC and MEK pathways. (A) Undifferentiated HL-60 cells are suspension cells and are washed out before crystal violet staining. Thus, no cells are stained on the dish. (B) TPA-differentiated HL-60 cells are attached cells and can be stained with crystal violet on the dish. (C) HL-60 cells are stained with crystal violet and the O.D. was determined at O.D. 550 nm in adhesion assay. Note that the O.D. value shows that only TPA-differentiated cells (TPA) can adhere on the dish, but undifferentiated (C), PD98059 (PD) and bisindolylmaleimide I (BI) pretreated cells can not adhere on the dish. Each data point was calculated from four triplicate groups and displayed as means ± S.D.

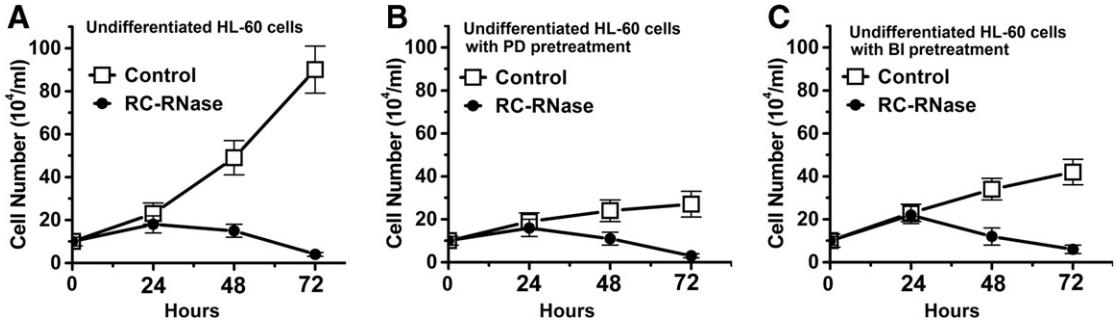

Fig. 2. RC-RNase induces cytotoxicity on undifferentiated cells. (A) RC-RNase induces cytotoxicity on undifferentiated HL-60 cells. (B) RC-RNase induces cytotoxicity on undifferentiated HL-60 cells with PD98059 (PD) pretreatment. (C) RC-RNase induces cytotoxicity on undifferentiated HL-60 cells with bisindolylmaleimide I (BI) pretreatment. Each data point was calculated from four triplicate groups and displayed as means ± S.D.

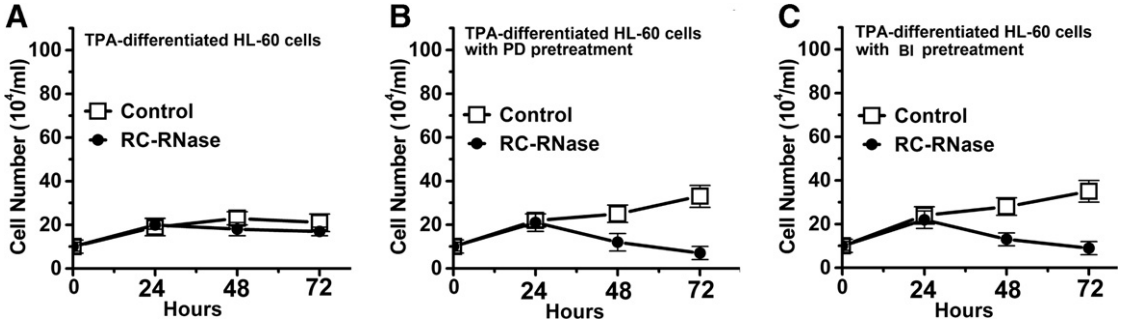

Fig. 3. TPA-differentiated cells inhibit RC-RNase-induced cytotoxicity through PKC and MEK pathways. (A) RC-RNase can not induce cytotoxicity on TPA-differentiated HL-60 cells. (B) RC-RNase induces cytotoxicity on TPA-differentiated HL-60 cells with PD98059 (PD) pretreatment. (C) RC-RNase induces cytotoxicity on TPA-differentiated cells HL-60 with bisindolylmaleimide I (BI) pretreatment. Each data point was calculated from four triplicate groups and displayed as means ± S.D.

ber significantly in TPA-differentiated cells (Fig. 3A). In contrast, the cell numbers in TPA-differentiated cells with bisindolylmaleimide I or PD98059 pretreatment decreased significantly (Fig. 3B and C). Furthermore, RC-RNase decreased the cell number in undifferentiated cells following bisindolylmaleimide I or PD98059 pretreatment (Fig. 2B and C). Summarised, our data shows that TPA-differentiated cells inhibit RC-RNase-induced cytotoxicity through the PKC and MEK pathways.

3.3. Caspase-9/-3 activation is inhibited on TPA-differentiated cells

After RC-RNase treatment, caspase-9/-3 activities are induced in undifferentiated cells but are inhibited in TPA-differentiated cells (Fig. 4). In addition, RC-RNase does not inhibit caspase-9/-3 activities on TPA-differentiated cells pretreated with bisindolylmaleimide I or PD98059 (Fig. 4). Our study also showed that if undifferentiated cells were pretreated with bisindolylmaleimide I or PD98059, then RC-RNase can not inhibit caspase-9/-3 activities on these cells (Fig. 4).

3.4. Caspase-9/-3 activation is inhibited on RA-differentiated cells

Undifferentiated cells and RA-differentiated cells can be easily determined by a NBT assay. RA-differentiated cells can reduce NBT and generate the black–blue formazan product. In this study, undifferentiated HL-60 cells (Fig. 5A) were successfully induced to differentiate into granulocytes with retinoic acid treatment (RA-differentiated cells) (Fig. 5B). The differentiation procedure was only inhibited if these cells were treated with bisindolylmaleimide I or PD98059 (Fig. 5C and D). Like TPA-differentiated cells, RA-differentiated cells can inhibit RC-RNase-induced caspase-9/-3-mediated cytotoxicity and cannot inhibit RC-RNase-induced caspase-9/-3-mediated cytotoxicity after pretreatment with PD98059 or bisindolylmaleimide I pretreatment (Fig. 6). Overall, our study shows that PKC and MEK pathways inhibit RC-RNase-induced caspase-9/-3-mediated cytotoxicity on TPA- and RA-differentiated cells.

4. Discussion

Previous studies showed that RC-RNase induce a strong cytotoxicity on poorly differentiated hepatoma cells but not on well-differentiated hepatoma cells [4]. Moreover, it has been demonstrated that RC-RNase induced cytotoxicity on undifferentiated cells but not on RA-differentiated cells [5]. Presently, this study demonstrates that TPA-differentiated cells can also inhibit RC-RNase-induced cytotoxicity (Fig. 3). These studies indicate that RC-RNase-induced cytotoxicity correlates with cell differentiation though the mechanisms remain unclear.

HL-60 cells can be induced to differentiate into granulocytes or into macrophage-like cells after pretreatment with RA and TPA, respectively [9,10]. This study also showed that PKC and MEK pathways could be activated on TPA- and RA-differentiated cells [10–12,18–21]. Therefore, we consider that differentiated cells inhibit RC-RNase-induced cytotoxicity through

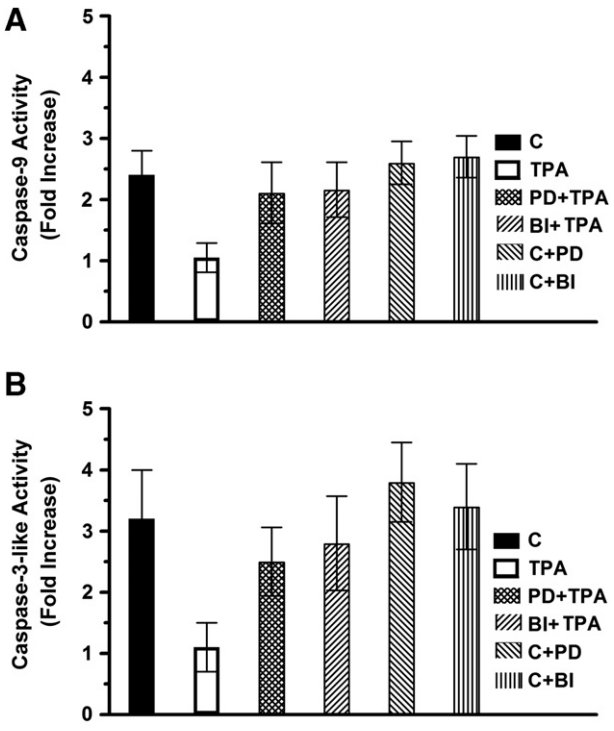

Fig. 4. PKC and MEK pathways inhibit RC-RNase-induced caspase-9/-3-mediated cytotoxicity on TPA-differentiated cells. (A) Caspase-9 and (B) caspase-3 activities are determined on different HL-60 cells with RC-RNase treatment for 72 h. Fold increase of caspases activities are determined as described in Section 2.6. Note cells used in the study are undifferentiated cells (C), TPA-differentiated cells (TPA), TPA-differentiated cells with PD98059 (TPA + PD) or bisindolylmaleimide I (TPA + BI) pretreatment and undifferentiated cells with PD98059 (C + PD) or bisindolylmaleimide I (TPA + BI) pretreatment. Only TPA-differentiated cells inhibit RC-RNase-induced caspases activation. Each data point was calculated from four triplicate groups and displayed as means ± S.D.

PKC and MEK pathways. To demonstrate this hypothesis, PKC and MEK inhibitors were used to block these pathways, which make the cells unable to inhibit RC-RNase-induced cytotoxicity. Our obtained data suggests that the PKC and MEK pathways can inhibit RC-RNase-induced cytotoxicity.

Previous studies showed that the p38 pathway is related to HL-60 cells differentiation [12]. To test whether the p38 pathway inhibits RC-RNase-induced cytotoxicity, a p38 inhibitor (SB202190) was used in this study. Results showed that RC-RNase cannot induce cytotoxicity on differentiated cells despite pretreatment with p38 inhibitor (data not shown). Thus, the p38 pathway cannot inhibit RC-RNase-induced cytotoxicity. Taken together, our study is the first to demonstrate that RC-RNase-induced cytotoxicity is inhibited through the PKC and MEK pathways but not through the p38 pathway.

Two major caspase-mediated cytotoxicity pathways have been described in previous studies. The death receptor pathway, in which caspase-8 is activated through cell surface receptors [22] and the mitochondrial death pathway, in which caspase-9 is activated through mitochondrial alterations [23]. It has been demonstrated before that the MEK pathway can

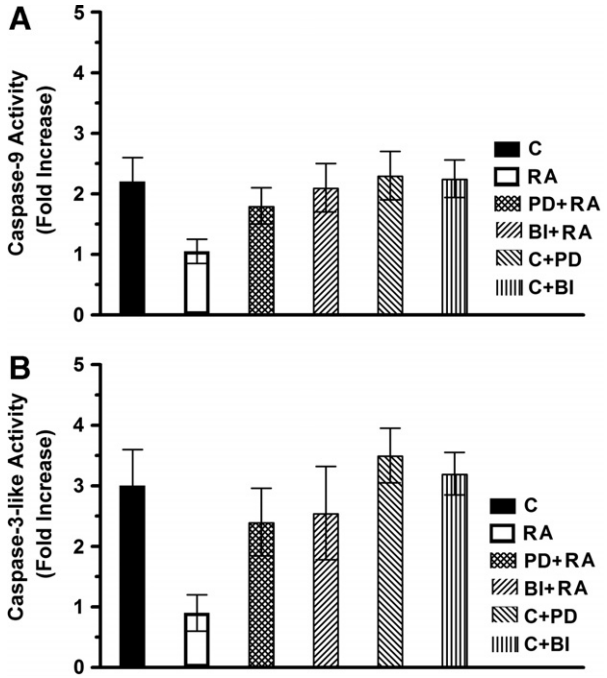

Fig. 6. PKC and MEK pathways inhibit RC-RNase-induced caspase-9/-3-mediated cytotoxicity on RA-differentiated cells. (A) Caspase-9 and (B) caspase-3 activities are determined on different HL-60 cells with RC-RNase treatment for 72 hours. Note: Cells used in the study are undifferentiated cells (C), RA-differentiated cells (RA), RA-differentiated cells with PD98059 (RA + PD) or bisindolylmaleimide I (RA + BI) pretreatment and undifferentiated cells with PD98059 (C + PD) or bisindolylmaleimide I (TPA + BI) pretreatment. Only RA-differentiated cells inhibit RC-RNase-induced caspases activation. Each data point was calculated from four triplicate groups and displayed as means ± S.D.

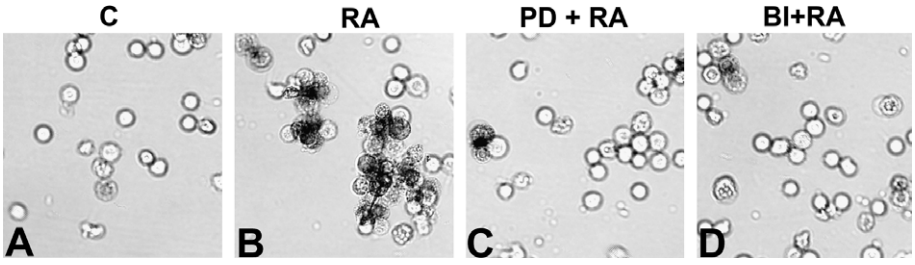

Fig. 5. RA-differentiated cells can reduce NBT and generate blue–black formazan. (A) Undifferentiated HL-60 cells. (B) HL-60 cells are induced to differentiate into granulocytes with RA treatment for 5 days (RA-differentiated cells). (C) HL-60 cells are pretreated with PD98059 before RA treatment (PD + RA). (D) HL-60 cells are pretreated with bisindolylmaleimide I before RA treatment (BI + RA). Note: Only RA-differentiated cells can reduce NBT to generate blue–black formazan.

inhibit caspase-8 activation [13,14]. However, whether the MEK pathway can inhibit caspase-9/-3 activation remains unclear. Our study is able to demonstrate that RC-RNase can not induce caspase-9/-3 activation on differentiated cells, but if the differentiated cells are pretreated with MEK inhibitor, RC-RNase is able to induce caspase-9/-3 activation (Fig. 4). The data indicate that the MEK pathway inhibits not only caspase-8 activation but also caspase-9/-3 activation. The data observed from this study further identified that RC-RNase can not induce caspase-9/-3 activation on differentiated cells unless the cells were pretreated with PKC inhibitor. In summary, our study is the first to indicate that both MEK and PCK pathways can inhibit caspase-9/-3 activation.

Acknowledgement: This work was sponsored by a Grant from the National Science Council, ROC (NSC 95-2320-B-241-007) and Tzu-Chi hospital (TCRD96-13 and TCRD-97-06).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.02.018.

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